Bacterial Production of Tetrodotoxin in Four Species of Chaetognatha

ERIK V. THUESEN[1] AND KAZUHIRO KOGURE

Ocean Research Institute, University of Tokyo, 1-15-1 Minamidai, Nakano-ku, Tokyo 164, Japan

Abstract. Tetrodotoxin (TTX) producing bacteria have been isolated from four species of planktonic chaetognaths: *Flaccisagitta lyra, Parasagitta elegans, Zonosagitta nagae,* and *Eukrohnia hamata.* TTX production in bacterial isolates from the four species of venomous chaetognath species was indicated by neuroblastoma cell culture bioassay and high performance liquid chromatography. A common species of marine bacterium, *Vibrio alginolyticus,* was identified as most likely responsible for the production of TTX. Extracellular TTX in the culture medium at concentrations of 280–790 pg/ml indicated that *V. alginolyticus* may be sufficiently capable of replenishing venom TTX within 24 h. It is suggested that this bacterium lives associated with these chaetognaths and is responsible for the production of the TTX in chaetognath venom.

Introduction

Observations of arrowworms paralyzing copepods in the laboratory (Parry, 1944; Nagasawa, 1985) and investigations of their raptorial morphology (Bieri *et al.,* 1983; Thuesen and Bieri, 1987) indicated that these primary carnivores use venom to enhance the likelihood of successful prey capture. Suppositions of their venomous nature were confirmed with the recent discovery that the sodium channel blocking agent tetrodotoxin (TTX) is present in the heads of a diverse number of chaetognaths (Thuesen *et al.,* 1988). Since many species of marine bacteria are evidently capable of producing TTX (Simidu *et al.,* 1987; Tamplin *et al.,* 1987; Yotsu *et al.* 1987), the present study was undertaken to determine if bacteria may be responsible for the production of TTX in chaetognath venom.

Materials and Methods

Zooplankton sampling was carried out on the *R/V Hakuho Maru,* Ocean Research Institute, University of Tokyo, cruise KH-87-4 in the Kuroshio and Oyashio mixing area off the northwestern coast of Japan. A 160-cm ORI net hauled obliquely to 1000 m and closing 56-cm MTD nets hauled horizontally at determined depth intervals to 700 m were used to collect samples. Animals were removed from the plankton sample immediately after collection, identified, and transferred to sterilized seawater. The chaetognaths *Flaccisagitta lyra* (Krohn), *Parasagitta elegans* (Verrill), *Zonosagitta nagae* (Alvariño), and *Eukrohnia hamata* (Möbius) were used as sources for the isolation of bacteria.

Immediately following removal from the plankton sample, chaetognaths were rinsed once more in sterilized seawater, decapitated, and the heads transferred to microhomogenizers. Heads were lightly homogenized either singly or in groups of five or ten. Some homogenates were prepared with the addition of one or two drops of sterilized seawater. Agar plates of $\frac{1}{5}$ strength ZoBell's 2216 medium (90% aged seawater) were then inoculated with the homogenate and incubated at 20°C. Since it is known that bacteria of the genus *Vibrio* are producers of TTX (Simidu *et al.,* 1987) and commonly associated with zooplankton (Simidu *et al.,* 1971), a survey of a variety of the bacterial colonies which had formed on the plates was carried out using the Hugh-Leifson test to select facultative anaerobes for analysis to determine TTX production.

Since all of the isolated *Vibrio* strains were found to have the property of "swarming on solid media," many of the possible *Vibrio* species were tentatively eliminated and the identification process simplified (Baumann and Schubert, 1984; West and Colwell, 1984). To identify the bacteria responsible for TTX production, we performed

the following tests: growth at 40°C, tolerance to 10% NaCl, utilization of sucrose, amylase production, gelatinase production, arginine dihydrolase, and Voges-Proskauer test of acetoin production. *Vibrio alginolyticus* (NCMB 1903) was used as a standard for comparison throughout the identification process.

Media for batch culture of bacteria was prepared of the following components: NaCl, 26.38 g; $MgSO_4 \cdot 7H_2O$, 2.46 g; K_2HPO_4, 1.0 g; Bacto-peptone (Difco), 5.0 g; yeast extract, 1.0 g; and distilled water, 1.0 l, adjusted to pH 7.5. Bacterial strains were inoculated into 250 ml of culture medium and incubated at 30°C with shaking until cells attained stationary growth phase (≈ 24 h). Cells were harvested by centrifugation at 7000 rpm and washed once with 0.3 M NaCl solution. The primary supernatant was adjusted to pH 5–6 with acetic acid, freeze-dried, and kept at −50°C. The pellet containing bacterial cells was resuspended with 10 ml 0.1% acetic acid and heated in a boiling water bath for 20 min to extract toxin. This solution was applied to centrifugation at 13,000 rpm for 15 min, and the resulting supernatant was freeze-dried and stored at −20°C. In preparation for HPLC, samples were reconstituted with distilled water, passed through 0.22-μm Millipore filters, and loaded into SEP-PAK C_{18} cartridges (Waters Associates). Samples were then eluted rapidly with 0.1% acetic acid, collected, and applied to HPLC.

A quick and simple bioassay employing mouse neuroblastoma cell culture (ATCC No. CCL 131) was recently developed by Kogure *et al.* (1988) which can detect picogram levels of sodium channel blocking neurotoxins. The biochemical basis of the assay is that veratridine enhances sodium ion influx in neuroblastoma cells when Na^+-K^+ ATPase is inhibited by ouabain and that TTX is able to effectively block this enhanced influx. The cell culture bioassay uses this cancellation of effects, and the presence of toxin in assay samples is demonstrated by cultures that remain viable despite the presence of veratridine and ouabain. Using TTX as a standard, the calculation of cell death ratios allows for the quantitative estimation of sample toxicity as shown by Kogure *et al.* (1988).

To ascertain that the sodium channel blocking toxin accumulated in bacterial cultures was TTX, ion-paired reverse-phase HPLC was carried out on a JASCO Tri Rotar-VI HPLC system with a 6×300 mm silica ODS column (Senshu Scientific Co.) following methods described previously (Nagashima *et al.*, 1987). The mobile phase consisted of 0.05 M phosphate buffer (pH 7.0) with 1% methanol and 2 mM heptanesulfonic acid delivered isocratically. The eluent was mixed with an equal volume of 3 N NaOH at 100°C and fluorescence was monitored at 381 nm excitation and 505 nm emission on a Hitachi F1000 fluorescence spectrophotometer. Three

microliters of TTX (Sigma) solution (0.1 mg/ml) was used as a standard. Although HPLC alone does not unequivocally prove the existence of TTX, it is a valid method for the confirmation of TTX in bacterial cultures given the results of Simidu *et al.* (1987), Tamplin *et al.* (1987), Yotsu *et al.* (1987), and sources cited therein.

Results

Bacterial colonies formed on all agar plates within a few days of incubation at 20°C. Swarming colonies were found on every plate regardless of the species of chaetognath or location of sample collection. On some plates this swarming colony dominated and no other colonies formed. These colonies were slightly arborescent, milky colored and were determined to be facultative anaerobes by the Hugh-Leifson test, designating them as members of the family Vibrionaceae. The majority of the other kinds of bacteria colonies tested negative for the Hugh-Leifson test and were excluded from the remaining testing procedures. Every *Vibrio* strain isolated from the heads of the chaetognaths *Flaccisagitta lyra, Parasagitta elegans, Zonosagitta nagae,* and *Eukrohnia hamata* was identified as *Vibrio alginolyticus* (Table I) following Baumann and Schubert (1984) and West and Colwell (1984).

Extracts taken from the batch cultures of all 15 *Vibrio* strains (Table I) effectively blocked the sodium channel as determined by the Kogure *et al.* (1988) cell culture bioassay. Within 24 h, extracellular toxin was present in the culture medium at concentrations ranging from 280 to 790 ng/ml (200 ng TTX equals one mouse unit) as estimated by cell culture bioassay. Ion-paired reverse-phase HPLC of both culture supernatant and bacterial cell extracts indicated that the responsible toxin was TTX (Fig. 1). Closely related TTX analogues have been shown to peak within several minutes after TTX using similar HPLC methods (Nagashima *et al.*, 1987; Yasumoto and Michishita, 1985), and these may also be present in the sample elution profile.

Discussion

Although Thuesen *et al.* (1988) found TTX in the heads of six species of chaetognaths, they did not determine the source of toxin production. The concentrations of extracellular TTX found in bacteria cultures indicates that *Vibrio alginolyticus* is capable of secreting TTX at substantial rates. This and the presence of *V. alginolyticus* in the heads of the arrowworms *Flaccisagitta lyra, Parasagitta elegans, Zonosagitta nagae,* and *Eukrohnia hamata* suggests that this bacterium is responsible for the production of the TTX in chaetognath venom. It is not yet known how many of the approximate 100 species of arrowworms use venom to capture prey, although the ecological diversity and morphological similarity of the

Table I

Characterization of the tetrodotoxin-producing bacteria of chaetognaths

Chaetognath	*Flaccisagitta lyra*	*Parasagitta elegans*	*Zonosagitta nagae*	*Eukrohnia hamata*	
Number of bacteria strains isolated	12	22	6	10	
Number of *Vibrio* strains (Hugh-Leifson test)	4	3	3	5	*Vibrio alginolyticus*
Chaetognath *Vibrio* characteristics					
Swarming	+	+	+	+	+
Growth at 40°C	+	+	+	+	+
Tolerance to 10% NaCl	+	+	+	+	+
Utilization of sucrose	+	+	+	+	+
Production of:					
Amylase	+	+	+	+	+
Gelatinase	+	+	+	+	+
Arginine dihydrolase	−	−	−	−	−
Acetoin (Voges-Proskauer)	+	+	+	+	+
Tetrodotoxin	+	+	+	+	+

known venomous species suggests that use of TTX venom may be very common throughout the phylum Chaetognatha (Thuesen *et al.*, 1988).

Figure 1. Ion-paired reverse phase HPLC elution profiles of tetrodotoxin standard (upper) and sample extract (lower) of the culture supernatant of *Vibrio alginolyticus*, which was isolated from the head of *Flaccisagitta lyra*. Some of the peaks following the TTX peak (arrows) within several minutes may possibly be related. less toxic tetrodotoxin analogues.

Of the six species of chaetognaths investigated previously (Thuesen *et al.*, 1988), *Parasagitta elegans* was found to have the highest toxicity: 320 pg of TTX per individual. The present study found *Vibrio alginolyticus* capable of secreting 280–790 pg of TTX in one microliter of culture medium within 24 h, figures that reasonably indicate that *V. alginolyticus* can replenish venom TTX over a necessary time scale. However, exact TTX secretion rates, numbers of *V. alginolyticus* inhabiting chaetognaths, and times required for replenishment of venom TTX following prey capture are not yet known.

The only other animal known to possess a TTX venom is the blue ringed octopus, *Hapalochlaena maculosa* (Scheumack *et al.*, 1978). TTX in the venom of *H. maculosa* is also apparently produced by bacteria (U. Simidu, pers. comm.). It has been suggested that *Vibrio alginolyticus* is the bacterium responsible for toxification of the tetraodontid fish, *Fugu vermicularis* (Noguchi *et al.*, 1987) as well as a toxic starfish, *Astropecten polyacanthus* (Narita *et al.*, 1987) and horseshoe crab, *Carcinoscorpius rotundicauda* (Kungsuwan *et al.*, in press). Our findings that *V. alginolyticus* can secrete TTX over a short time scale at relatively potent concentrations strengthens the argument for bacterial production of TTX in these animals. We consider *V. alginolyticus* to be the most apparent source of the TTX in chaetognath venom, albeit possible that venom TTX is produced by another as yet unidentified microorganism. However, it is remarkable that this identical TTX-producing bacterium has been found in association with such a wide array of marine animals which possess TTX.

Investigations of chaetognath-bacterial interactions are few. Unidentified bacteria have been reported as
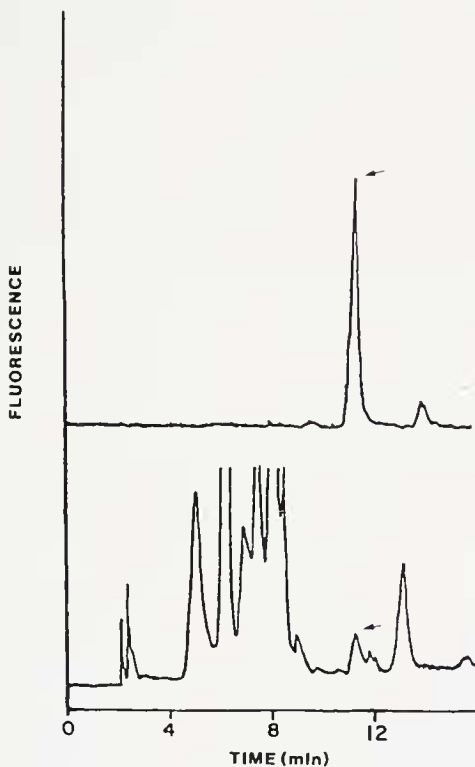
pathogens of chaetognaths both in the natural environment and in the laboratory (Nagasawa and Nemoto, 1984; Nagasawa *et al.*, 1984, 1985). Nair *et al.* (1988) investigated the bacterial flora of the neritic arrowworm *Aidanosagitta crassa* and found that *Vibrio* accounted for 12.7% of the bacteria associated with healthy individuals and 36.3% of those associated with weak and moribund specimens. These investigations were not developed further and identification of bacteria at the species level was not carried out. It is likely that *V. alginolyticus* accounted for some of the *Vibrio* species found by Nair *et al.* (1988), since all the *Vibrio* isolated in the present study were this species.

The exact location of *Vibrio alginolyticus* and the mechanism of accumulation of TTX in chaetognath heads is not yet known. Bieri *et al.* (1983) suggested that venom may be secreted from the vestibular papillae that lie beneath the tips of the posterior teeth, and this could be a sight of symbiosis. The vestibular pit, mouth, and gut are other possible locations of bacteria. The ultrastructural characteristics of bacterial associations has not yet been revealed in any of the animals that accumulate TTX, and it is not yet known if bacteria are enclosed within bacteriocytes or live within intercellular space. The role that TTX plays in the physiology of marine bacteria is still unknown, however its ability to stop the flow of sodium ions through cell membranes suggests that it may function in the regulation of ion exchange.

Acknowledgments

Literature Cited

Baumann, P., and R. H. W. Schubert. 1984. Family II. Vibrionaceae Veron 1965. Pp. 516–550 in *Bergey's Manual of Systemic Bacteriology*, N. R. Krieg and J. G. Holt, eds. Williams & Wilkins, Baltimore.

Bieri, R., D. Bonilla, and F. Arcos. 1983. Function of the teeth and vestibular ridge in the Chaetognatha as indicated by scanning electron microscope and other observations. *Proc. Biol. Soc. Wash.* 96: 110–114.

Kogure, K., M. Tamplin, U. Simidu, and R. R. Colwell. 1988. A tissue culture assay for tetrodotoxin, saxitoxin and related toxins. *Toxicon* 26: 191–197.

Kungsuwan, A., T. Noguchi, U. Simidu, K. Tsukamoto, Y. Shida, and K. Hashimoto. Tetrodotoxin-producing bacteria from the horseshoe crab *Carcinoscorpius rotundicauda. Toxicon* (In press).

Nagasawa, S. 1985. The digestive efficiency of the chaetognath *Sagitta crassa* Tokioka, with observations on the feeding process. *J. Exp. Mar. Biol. Ecol.* 87: 67–75.

Nagasawa, S., and T. Nemoto. 1984. X-diseases in the chaetognath *Sagitta crassa. Helgol. Meeresunters.* 37: 139–148.

Nagasawa, S., T. Nemoto, and U. Simidu. 1984. Bacterial invasions of chaetognaths under laboratory and natural conditions. *J. Oceanogr. Soc. Jpn.* 40: 327–333.

Nagasawa, S., T. Nemoto, and U. Simidu. 1985. Ecological aspects of deformed chaetognaths and visual observations of their periphytes. *Mar. Biol.* 87: 67–75.

Nagashima, Y., J. Maruyama, T. Noguchi, and K. Hashimoto. 1987. Analysis of paralytic shellfish poison and tetrodotoxin by ion-pairing high performance liquid chromatography. *Nippon Suisan Gakkaishi* 53: 819–823.

Nair, S., K. Kita-Tsukamoto, and U. Simidu. 1988. Bacterial flora of healthy and abnormal chaetognaths. *Nippon Suisan Gakkaishi* 54: 491–496.

Narita, H., S. Matsubara, N. Miwa, S. Akahane, M. Murakami, T. Goto, M. Nara, T. Noguchi, T. Saito, Y. Shida, and K. Hashimoto. 1987. *Vibrio alginolyticus*, a TTX-producing bacterium isolated from the starfish *Astropecten polyacanthus. Nippon Suisan Gakkaishi* 53: 617–621.

Noguchi, T., D. F. Hwang, O. Arakawa, H. Sugita, Y. Deguchi, Y. Shida, and K. Hashimoto. 1987. *Vibrio alginolyticus*, a tetrodotoxin producing bacterium, in the intestines of the fish *Fugu vermicularis vermicularis. Mar. Biol.* 94: 625–630.

Parry, D. A. 1944. Structure and function of the gut in *Spadella cephaloptera* and *Sagitta setosa. J. Mar. Biol. Assoc. U. K.* 26: 16–36.

Sheumack, D. D., M. E. H. Howden, I. Spence, and R. J. Quinn. 1978. Maculotoxin: a neurotoxin from the venom glands of the octopus *Hapalochlaena maculosa* identified as tetrodotoxin. *Science* 199: 188–189.

Simidu, U., K. Ashino, and E. Kaneko. 1971. Bacterial flora of phyto- and zoo-plankton in the inshore water of Japan. *Can. J. Microbiol.* 17: 1157–1160.

Simidu, U., T. Noguchi, D. F. Hwang, Y. Shida, and K. Hashimoto. 1987. Marine bacteria which produce tetrodotoxin. *Appl. Env. Microbiol.* 53: 1714–1715.

Tamplin, M. L., R. R. Colwell, S. Hall, K. Kogure, and G. R. Strichartz. 1987. Sodium channel inhibitors produced by enteropathogenic *Vibrio cholerae* and *Aeromonas hydrophila. Lancet* 1987-1: 975.

Thuesen, E. V., and R. Bieri. 1987. Tooth structure and buccal pores in the chaetognath *Flaccisagitta hexaptera* and their relation to the capture of fish larvae and copepods. *Can. J. Zool.* 65: 181–187.

Thuesen, E. V., K. Kogure, K. Hashimoto, and T. Nemoto. 1988. Poison arrowworms: a tetrodotoxin venom in the marine phylum Chaetognatha. *J. Exp. Mar. Biol. Ecol.* 116: 249–256.

West, P. A., and R. R. Colwell. 1984. Identification and classification of Vibrionaceae—an overview. Pp. 285–363 in *Vibrios in the Environment*, R. R. Colwell, ed. John Wiley & Sons, New York.

Yasumoto, T., and T. Michishita. 1985. Fluorometric determination of tetrodotoxin by high performance liquid chromatography. *Agric. Biol. Chem.* 49: 3077–3080.

Yotsu, M., T. Yamazaki, Y. Meguro, A. Endo, M. Murata, H. Naoki, and T. Yasumoto. 1987. Production of tetrodotoxin and its derivatives by *Pseudomonas* sp. isolated from the skin of a pufferfish. *Toxicon* 25: 225–228.